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In Vitro Effect on Human Leukemic K562 Cells of Co-Administration of Liposome-Associated Retinoids and Cytosine Arabinoside (Ara-C)

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The administration of retinoids has been demonstrated to be of potential utility in the therapy of a wide spectrum of neoplastic pathologies due to the ability to induce differentiation in a large variety of primary tumor cells as well as in vitro cultured cell lines. Moreover, a number of compounds, including hemin, cytosine arabinoside, and 5-azacytidine are able to induce erythroid differentiation of the erythroleukemic cell line K562. In this paper we determined whether a combined treatment of K562 cells with suboptimal concentrations of cytosine arabinoside and retinoids containing liposomes lead to a full expression of differentiated functions. Liposomes were prepared by reverse phase evaporation technique followed by extrusion through polycarbonate filters. Cell growth kinetics studies and intracellular detection of hemoglobin by benzidine staining were performed. The results obtained showed that the combined treatment with liposomes containing retinoids and sub-optimal concentration of ara-C is an effective strategy to induce K562 cell differentiation, minimizing at the same time toxic effects. Control experiments aimed to determine possible selection of subpopulations of K562 cells suggest that the observed results are not related to toxicity and/or potential selection of induced cells. In conclusion, liposomally delivered retinoids could be proposed for differentiation therapy as an effective strategy in the treatment and management of malignancy. In addition, the finding that liposomally delivered retinoids increase the capacity of cytosine arabinoside to induce erythroid differentiation, could be of interest in studies aimed at the development of treatment able to reactivate fetal globin genes in β-thalassemia patients. Am. J. Hematol. 62:33–43, 1999. © 1999 Wiley-Liss, Inc.

Key words: 1-β-D-arabinofuranosylcitosine; erythroid differentiation; ATRA; retinol; liposomes

INTRODUCTION

In the wide spectrum of biological response modifiers available for cancer chemotherapy, retinoids identify a family of compounds useful for a number of neoplastic pathologies [1–7]. The main advantage in the therapeutic use of retinoids, contrasting to most of the conventional chemotherapeutic agents, is represented by inhibition of the growth of cancer cells associated with induction of differentiated function rather than killing effect. Several retinoids are indeed able to influence the growth and the differentiation of malignant cells [8,9]. For instance, retinoids were found important regulators of cervical epithelial cell differentiation and have been used in the treatment of cervical cancer [8]. Recent data in addition demonstrated that treatment of acute promyelocitic leukemia by all-trans retinoic acid (ATRA) is the first model of differentiation therapy allowing achievement of more than 90% complete remission [8,9]. As an example, the oral retinoid formulation of ATRA has been recently approved by the FDA as therapeutic treatment for acute promyelocytic leukemia. Additional clinical studies suggested the use of a lipid-based ATRA formulation (namely Atragen®, Aronex Pharmaceuticals) in the treatment of AIDS related pathologies such as Kaposi' sar-

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coma [10,11]. In addition, the combined administration of IFN α-2b and retinoic acid has been demonstrated to affect proliferation and gene expression of human cervical carcinoma cells, thus representing a promising therapy for patients with advanced squamous cell cancer of both cervix and skin [11].

In spite of these interesting pharmacological effects, the hydrophobic nature of retinoids makes difficult their administration by both intravenous or oral route, requiring the utilisation of oily formulations or surfactant containing aqueous solutions. It is clear that the use of drug delivery systems, such as liposomes, might solve or greatly attenuate most of the problems associated with the systemic use of these drugs, increasing the solubility in water and possibly the plasma half-life of retinoids.

In addition, a recently published paper from our laboratory reported the effects of the combined treatment of retinoids and suboptimal concentrations of cytosine arabinoside on K562 cells [12]. The results obtained on cell differentiation showed that ATRA and retinol (Ret-OH), when used alone at sub-optimal concentration up to 40 μM, are not able to induce erythroid differentiation of K562 cells, whilst when 40 μM ATRA or Ret-OH are combined with suboptimal concentration of ara-C, erythroid differentiation of K562 cells is readly achieved [12]. These results indicated that retinoids and ara-C exert a cooperative effect on erythroid differentiation when used at suboptimal concentrations, not able per se to induce either differentiation nor block of cell growth.

In the present paper, the differentiating activity of ara-C co-administered with liposome-associated ATRA and Ret-OH on K562 cell line was investigated.

MATERIALS AND METHODS

Chemicals

Egg phosphatidyl choline was purchased from Lipid Products (Surrey, UK). Cholesterol (CH), ATRA, and Ret-OH were obtained from Fluka (Buchs, Switzerland). 1-β-D-arabinofuranosyl-cytosine (ara-C) was from Sigma (St. Louis, MO). All other materials and solvents in the high microbial purity grade were from Sigma.

Liposome Preparation

0.5 ml of a chloroform-methanol solution (2:1, v/v) containing 50 mg of egg phosphatidyl choline, 0.1 mg of CH and ATRA or Ret-OH at the final molar ratio of 67:33:4, were placed in a 25 ml round bottom flask. The mixture was then vacuum-dried under nitrogen using a rotary evaporator. The resulting dried lipid-retinoid mixed film was dissolved in 4 ml of diethyl ether; to this solution 1 ml of isotonic Palitzsch (IPB) buffer pH 7.4 (5 mM $Na_2B_4O_7$, 180 mM H_3BO_3, 18 mM NaCl) was added. Afterwards, the buffer-lipid-retinoid mixture was sonicated at 0°C for 10 min in a bath sonicator in order to obtain a stable emulsion. The ether present in the emulsion was removed at room temperature by rotary evaporation under reduced pressure to give a turbid liposome dispersion.

The liposome dispersion was diluted with borate buffer to 2 ml final volume and then extruded through two stacked standard 25 mm diameter polycarbonate filters with calibrated pore size (Nucleopore, Pleasanton, CA). The nitrogen pressure for the extrusion of the vesicles was 10–20 bars. In order to obtain a separation of the free retinoid from liposomes, a gel filtration on Sepharose 4B column (Pharmacia, Uppsala, Sweden; 1.5 cm diameter, 50 cm length) was performed. The column was pre-equilibrated and eluted with IPB. Each fraction collected from Sepharose 4B column was subjected to ultraviolet/Vis determination either in buffer or in ethanol. The turbidity of the suspension obtained by the light scattering measurement in buffer gives rise to the presence of liposomes. Whereas, the ultraviolet/Vis spectrum measured in ethanol (at 343 nm and 324 nm for ATRA and Ret-OH, respectively) allows the determination of the retinoid concentration by mean of a previously constructed calibration curve. In ethanol in fact the macromolecular assembly of liposomes is completely solubilized and the obtained phosphatidyl choline do not show any absorption band in the spectral region (310–350 nm) used for retinoid quantitation.

Vesicles produced by extrusion through 100 nm pore-size membranes were called VET_{100}(retinoids), where VET indicates vesicles produced by extrusion techniques and the subscript number the pore size of the used polycarbonate filter [13]. In round brackets is indicated the type of retinoid present in the formulation. It is to be underlined that in the case of retinoids, the lipophylic molecules are intercalated within the vesicle phospholipid bilayer rather than encapsulated in the internal aqueous space as in conventional liposomes.

Liposome Morphology

Liposome shape and surface characteristics were studied by freeze fracture electron microscopy. After freezing the sample by propane jet technique, the cryofixed preparation was fractured at 108 K in a Balzen BAF 300 at 10^{-5} Pa. Photomicrographs were taken on Agfa Scientia 23D56 cut films and developed in Geratone G5C for 3.5 min at 293 K.

Liposome size and size distribution were determined with an automated, laser-based submicron particle size analyzer Zetasizer 3000 PCS (Malvern Instruments, UK) equipped with a 5 mW helium neon laser. All liposome dispersions were made up using a double distilled and sterilized water for injections. Water (3 ml) was placed in the tube and 50 ml of liposome dispersion were added. The measurement were made at 25°C at an angle of 90°. Data were interpreted by using Contin software.

In Vitro Dialysis

The determination of the in vitro release of ATRA and Ret-OH from liposome was determined by dialysis method. Briefly, 2 ml of liposome suspension (100 mg/ml of lipids) were poured into a dialysis tube (molecular weight cut off 10,000–12,000; Medi Cell International, UK). Dialysis tubes were then alternatively placed into 30 ml of IPB, IPB:methanol (9:1 v/v), IPB:foetal calf serum (9:1 v/v) or empty liposome suspension (100 mg/ml of phospholipids) in a horizontal shaker. Afterwards, samples were withdrawn at regular time intervals from the receiving buffer up to 24 hr. The amount of released retinoids was determined by reverse-phase high-performance liquid chromatography using an high-performance liquid chromatographic system consisting of a Bruker LC21-C chromatographic pump (Bruker, Germany), a Rheodyne 7125 sample injection valve (Rheodyne; equipped with a 100 ml loop), and a Chrom-A-Scope rapid scan ultraviolet detector (Carlo Erba Strumentazione, Italy). Samples were chromatographed on a 150 × 4.6 mm reverse-phase stainless steel column packed with 5 mm particles (Model LC-18-DB, Supelco), eluted isocratically at room temperature with a mobile phase constituted of 180 mM ammonium acetate (pH 3.0)/methanol (4:96, v/v) at a flow rate of 1 ml/min. For each retinoid was constructed a calibration curve from standard solutions. Retinoids detection was monitored at the γ_{max} characteristic of each compound.

Cell Lines and Culture Conditions

The human myeloid erythroid leukaemia K562 cells [14,15] were maintained in RPMI 1640 (GIBCO, Burlington, Ontario, Canada) in 10% fetal calf serum (GIBCO), 5% CO_2 supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin. Because retinoids display a very low water solubility, the compounds were added to cell medium as ethanolic solution. In each induction experiment, K562 cells were usually seeded at a concentration of 1.2×10^4 cells/ml. Control cell populations were treated with an equivalent amount of ethanol; this treatment shows no effect on K562 cell growth and differentiation. Cell growth was determined by counting the cell number/ml after different days of in vitro cell culture using a Model ZF Coulter Counter (Coulter Electronics, Hielah, FL). Cell viability was determined by the trypan blue exclusion test [14].

Hemoglobin Determination

K562 cells containing heme or hemoglobin were detected by specific reaction with a benzidine/hydrogen peroxide solution as reported elsewhere [16]. Briefly, 5 μl of K562 cells were added to 5 μl of the benzidine stain solution (2% benzidine in 0.5 M glacial acetic acid brought to 1% hydrogen peroxide just before assay) and the proportion of benzidine-positive cells was determined by using an inverted microscope.

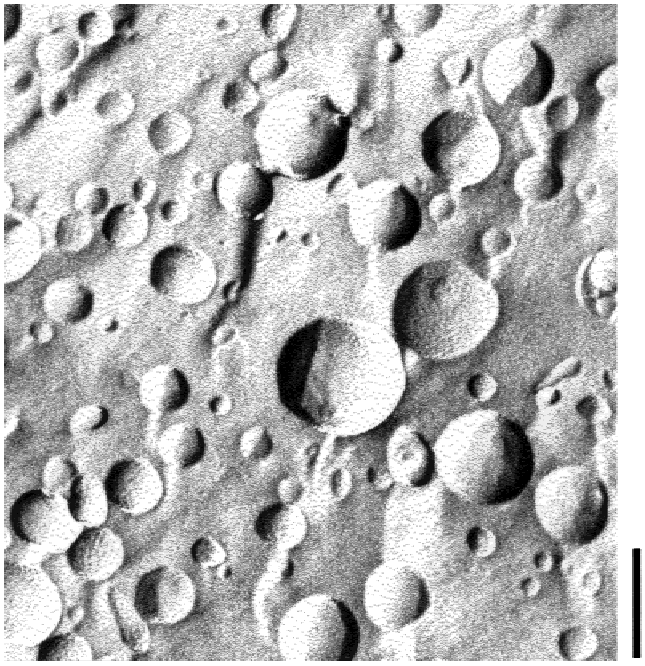

Fig. 1. Freeze fracture electron microscopy of VET_{100}(ATRA). Liposomes were prepared by reverse-phase evaporation method followed by five extrusion cycles through polycarbonate filters with 100 nm pore size. Scale bar = 100 nm.

RESULTS AND DISCUSSION

Preparation and Characterization of Liposome-Associated Retinoids

Liposome-associated retinoic acid [VET_{100}(ATRA)] and liposome-associated Ret-OH [VET_{100}] were prepared by reverse phase evaporation [17] followed by five extrusion cycles through two stacked polycarbonate membranes [13] with 100 nm pores. The extrusion step was performed in order to obtain unilamellar liposomes with an homogeneous size distribution as confirmed by the freeze-fracture electron microphotographic analysis reported in Figure 1, where the VET_{100}(ATRA) taken as example were depicted. The liposome size was determined by photon correlation spectroscopy. In Table I the mean diameters expressed by intensity, volume and number of liposomes before and after extrusion are reported.

In order to separate the free drug from the liposome-associated drug a gel-permeation chromatography was performed. As clearly appreciable from the elution profiles reported in Figure 2, both the chromatograms showed two peaks. The first peak was referred to the elution of VET_{100}(retinoid), while the second smaller peak was attributed to the elution of the free drug. The presence of liposomes in the first peak was indicated by

TABLE I. Photon Correlation Spectroscopy Analysis of Liposome-Associated Retinoids

Liposome	Subpopulation	Relative %	Mean diameter
By intensity			
Not extruded (ATRA)	1	59.2	96.1
	2	40.8	376.5
VET_{100}(ATRA)	1	100	126.6
Not extruded (Ret-OH)	1	100	180.8
VET_{100}(Ret-OH)	1	100	153.4
By volume			
Not extruded (ATRA)	1	66.2	83.7
	2	33.8	428.0
VET_{100}(ATRA)	1	100	106.7
Not extruded (Ret-OH)	1	78.6	84.7
	2	21.4	428.3
VET_{100}(Ret-OH)	1	100	118.9
By number			
Not extruded (ATRA)	1	99.8	75.2
	2	0.2	402.2
VET_{100}(ATRA)	1	100	92.1
Not extruded (Ret-OH)	1	99.8	81.8
	2	0.2	402.7
VET_{100} (Ret-OH)	1	100	96.8

the turbidity of the solution and by photon correlation spectroscopy analysis. On the other side, the presence of retinoids in both peaks was demonstrated by spectrophotometric analysis. The liposome association yield of ATRA and Ret-OH was 91% and 34%, respectively.

The selection of liposomes constituents was made in consideration of their stability in tissue culture conditions and eventually in blood after in vivo administration. In order to keep the liposome composition as simple as possible facilitating the preformulatory studies, pure phosphatidyl choline or phosphatidyl choline/cholesterol (67:33, mol:mol) liposomes were selected. Liposomes constituted of phosphatidyl choline/cholesterol and those composed of sole phosphatidyl choline were found almost superimposable in term of pharmaceutical characteristics such as size, elution profiles, retinoid association yields and in vitro activity (data not shown). In spite of these similarities, the release or exchangeability of retinoids from cholesterol free or cholesterol containing liposomes were determined and compared. To this aim a series of in vitro dialysis experiments were performed and the obtained results are summarized in Table II. The data indicate that the association complex between retinoids and liposomes is quite stable. VET_{100}(ATRA) were exposed to different receiving dialysis media based on isotonic Palitzsch buffer, namely (a) pure buffer, (b) buffer plus 10% methanol, (c) buffer plus 10% foetal calf serum or (d) empty liposome suspensions. Particularly, the addition of foetal calf serum was considered in order to evaluate the liposome stability in the presence of serum proteins and to simulate the in vitro cell culture medium; whilst empty liposomes were used in order to mimic cell membranes to evaluate exchangeability of

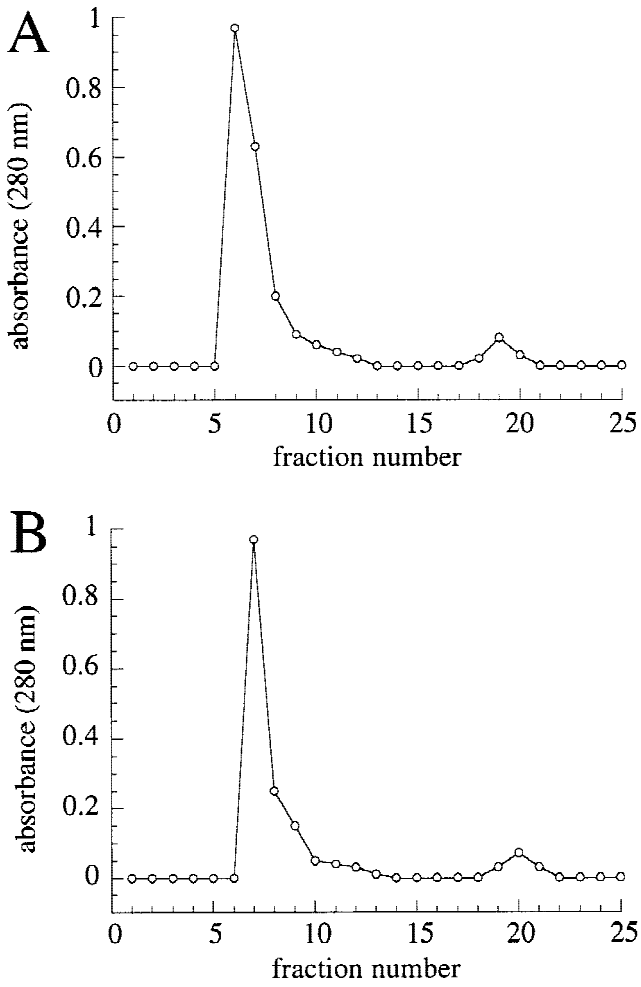

Fig. 2. Elution profiles of VET_{100}(retinoids). Gel-permeation chromatography was performed on a Sepharose 4B column (1.5 cm diameter; 50 cm length; flow rate: 420 ml/min; 8.4 ml/fraction). A: VET_{100}(ATRA); B: VET_{100}(Ret-OH).

TABLE II. Release or Exchangeability Properties of ATRA from Phosphatidyl Choline or Phosphatidyl Choline/Cholesterol Liposomes*

	Released or exchanged ATRA (%)[a]	
Dialysis solution	PC liposomes	PC/Chol liposomes
IPB	10.7	10.1
IPB + 10% methanol	12.6	7.6
IPB + 10% foetal calf serum	10.6	14.1
IPB + empty liposomes	37.0	28.7

*PC = phosphatidyl choline; PC/Chol = phosphatidyl choline/cholesterol (67:33 mol/mol); IPB = isotonic Palitzsch buffer.

[a]Experimental conditions are reported in the Materials and Methods section.

ATRA among membranes. After 24 hr of incubation in the presence of the above reported media, the percentage of ATRA associated to liposomes was found above 60% in all cases, without significative differences between

TABLE III. Effect of Free and Liposome-Associated ATRA and Retinol on Both Cell Growth and Differentiation of Human Erythroleukemic K562 Cells

	IC_{50} (μM)[a]		
Compound	Free	Liposome	G.I.P.[b]
All-trans retinoic acid	50.0	0.76	65.8
ATRA (37.5 nM ara-C)	38.0	0.8	47.5
ATRA (75 nM ara-C)	31.0	1.3	25.8
Retinol	35.3	0.66	51.5
Ret-OH (37.5 nM ara-C)	33.0	0.40	82.5
Ret-OH (75 nM ara-C)	38.0	0.60	63.3
	DC_{50} (μM)[c]		
	Free	Liposome	G.I.D.[d]
All-trans retinoic acid			
ATRA (37.5 nM ara-C)	5.2	1.26	10.4
ATRA (75 nM ara-C)	2.6	0.63	3.6
Retinol			
Ret-OH (37.5 nM ara-C)	24.9	0.97	22.4
Ret-OH (75 nM ara-C)	5.2	1.16	7.5

[a]Inhibitory concentration 50%: compound concentration (μM) required to cause a 50% inhibition of in vitro growth of K562 cells.
[b]Gain Index Proliferation: ratio IC_{50}(free-retinoid)/IC_{50}(lipo-retinoid).
[c]Differentiating concentration 50%: compound concentration (μM) required to cause a 50% differentiation of in vitro K562 cells.
[d]Gain Index Differentiation: ratio DD_{50}(free-retinoid)/DC_{50}(lipo-retinoid).
Data represent the average of three independent experiments. Determinations were performed after 7 days of cultured cells.

cholesterol free or cholesterol containing vesicles. The highest percentage of release was found in the case of incubation with empty liposomes in reason of the known ability of retinoids to easily transfer between biological membranes [18].

Antiproliferative Activity of Liposome-Associated Retinoids

The in vitro antiproliferative activity of VET_{100}(ATRA) and VET_{100}(Ret-OH) was determined and compared with that of the corresponding free retinoids by treating K562 cells with the same amount of drugs. It is well known that the length of cell cycle of K562 cells is 22–24 hr [19]. Therefore, in order to avoid that the concentration of cells per ml reaches plateau levels before 5–6 days of cell culture, K562 cells were seeded at the initial concentration of 2×10^4 cells/ml.

The obtained results are reported in Table III and Figure 3 and indicate that VET_{100}(retinoids) displayed, respectively, a 65- and 51-fold higher antiproliferative activity towards K562 cells with respect to the free ATRA (Fig. 3A) and Ret-OH (Fig. 3B). These data suggest that the augmented effect of retinoids could be reasonably due to the increased solubility and/or bioavailability of the liposomally delivered compounds.

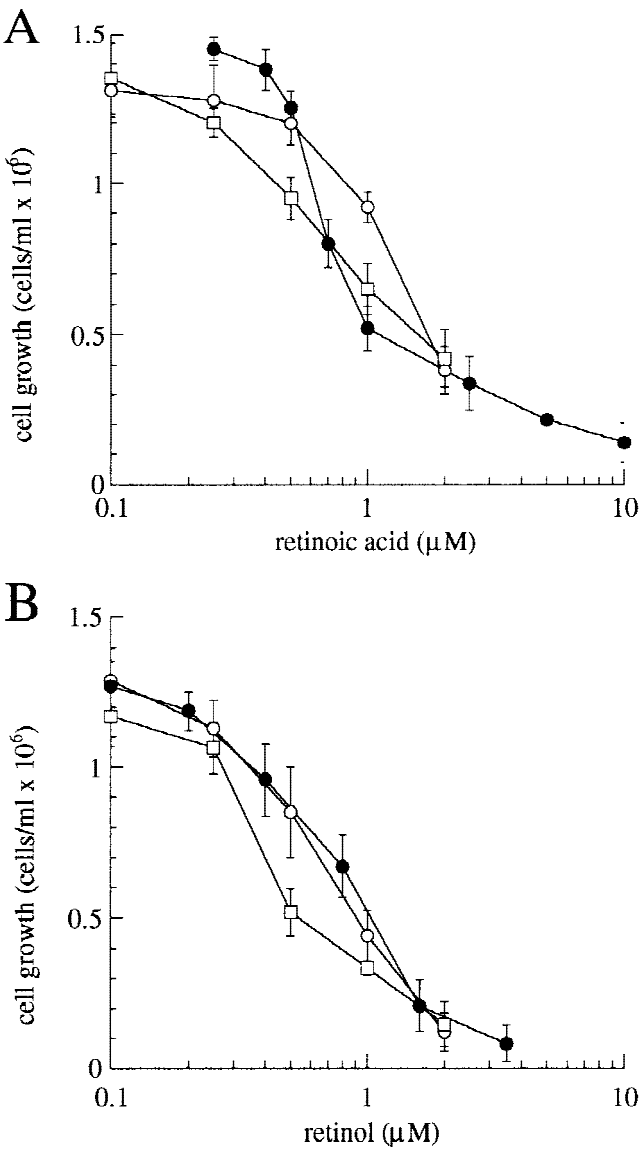

Fig. 3. Effects of VET_{100}(retinoids) and ara-C on cell growth of K562 cells. A: VET_{100}(ATRA) activity on proliferation of K562 cells incubated in the absence (●) or in the presence of 37 (□) and 75 nM (○) ara-C. B: VET_{100}(Ret-OH) activity on proliferation of K562 cells incubated in the absence (●) or in the presence of 37 (□) and 75 nM (○) ara-C. As control, cells were also treated with the same amount of empty liposomes. This treatment did not lead to any modification of cell growth kinetics with respect to untreated cells [12].

Antiproliferative Effects of the Combined Administration of Liposome-Associated Retinoids and Ara-C

Free and VET_{100}(retinoids) were employed in combination with sub-optimal concentrations of ara-C, namely 18.25, 37.5, and 75 nM. These concentrations were selected on the basis of previously published experiments demonstrating that 18.25, 37.5, and 75 nM of ara-C used

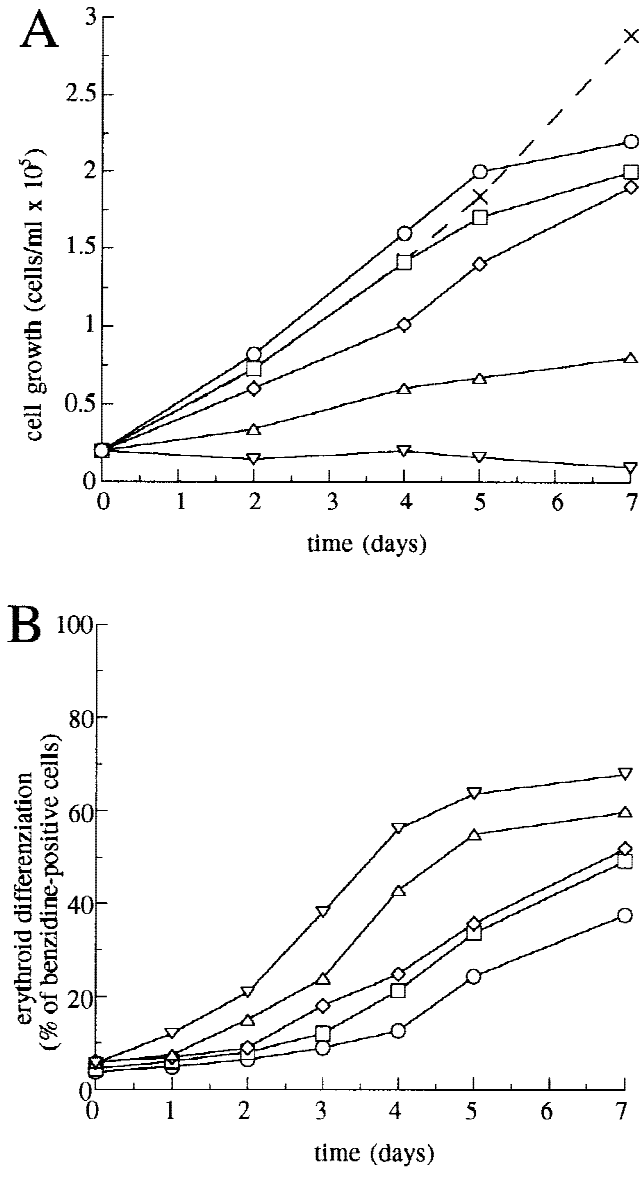

Fig. 4. A: Growth curves of K562 erythroleukemic cells treated with ara-C 18.25 nM (○), 37.5 nM (□), 75 nM (◇), 1 μM (△) and 5 μM (▽). For comparison the kinetics of cell growth of untreated cells is reported (X). B: Kinetics of ara-C mediated induction of K562 erythroid differentiation. K562 cells were cultured in the presence of 18.25 nM (○), 37.5 nM (□) and 75 nM (◇), 1 μM (△) and 5 μM (▽) cytosine arabinoside. For comparison the kinetics of cell differentiation of untreated cells is reported (X).

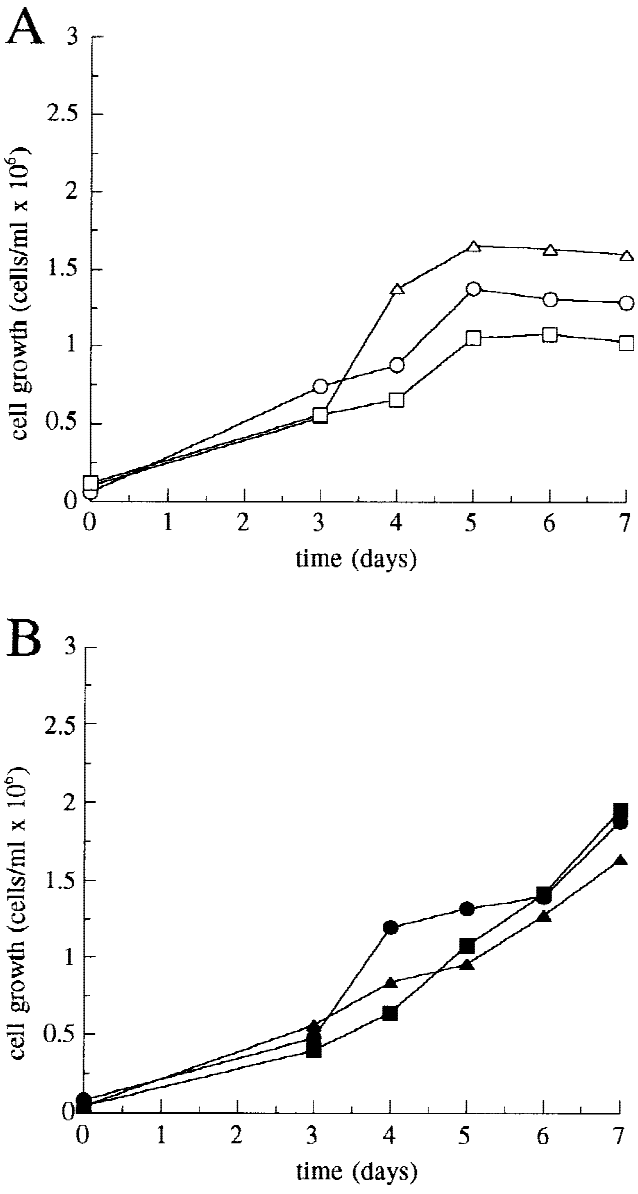

Fig. 5. Growth curves of K562 cells simultaneously treated with free ATRA plus ara-C (A) or VET_{100}(ATRA) plus ara-C (B). A: 40 μM free ATRA plus 18.25 nM ara-C (○); 40 μM free ATRA plus 37.5 nM ara-C (□); 40 μM free ATRA (△). B: 0.025 μM VET_{100}(ATRA) plus 18.25 nM ara-C (●); 0.025 μM VET_{100}(ATRA) plus 37.5 nM (■) ara-C; 0.025 μM VET_{100}(ATRA) (▲).

alone caused only 8% and 16% reduction of cell growth, respectively after 7 days of cell culture [12]. These results are also confirmed by the data obtained from the experiments reported in Figure 4, which demonstrate that concentrations of ara-C below 37.5 nM are not able to affect both growth (Fig. 4A) and differentiation (Fig. 4B) of K562 cells. The initial concentration of K562 cells was 2×10^4 cells/ml. Figure 5A shows a representative experiment demonstrating that 40 μM ATRA alone or in the presence of 18.25 or 37.5 nM ara-C inhibit K562 cell proliferation. When K562 cells were cultured with 0.025 μM VET_{100}(ATRA), alone or in the presence of 18.25 or 37.5 nM ara-C (Fig. 5B), no major differences in the cell growth kinetics were obtained. Superimposable results were obtained in the case of liposome containing Ret-OH (see Fig. 6).

The complete set of the obtained results is reported in Table III, which shows that the presence of 37.5 and

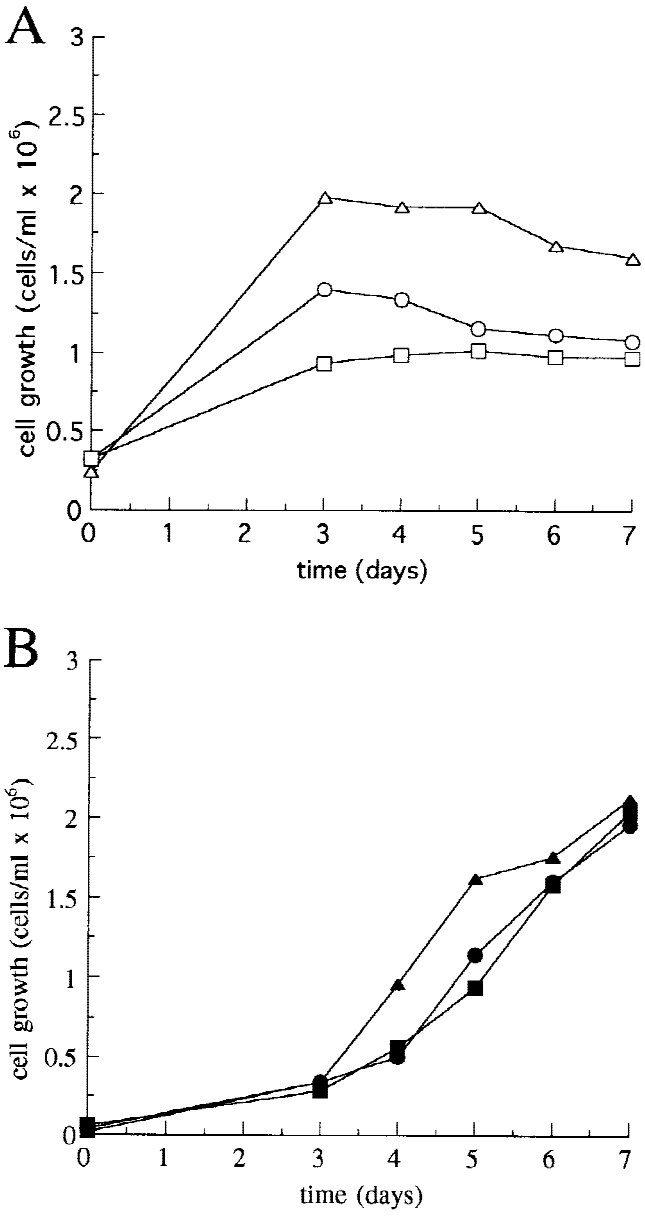

Fig. 6. Growth curves of K562 cells simultaneously treated with free Ret-OH plus ara-C (A) or VET_{100}(Ret-OH) plus ara-C (B). A: 40 μM free Ret-OH plus 18.25 nM ara-C (○); 40 μM free Ret-OH plus 37.5 nM ara-C (□); 40 μM free Ret-OH (△) B: 0.025 μM VET_{100}(Ret-OH) plus 18.25 nM ara-C (●); 0.025 μM VET_{100}(Ret-OH) plus 37.5 nM (■) ara-C; 0.025 μM VET_{100}(Ret-OH) (▲).

75 nM ara-C in combination with retinoids leads to a slight reduction in the IC_{50} value (dose inhibiting the growth of the 50% of the cells). The IC_{50} of ATRA alone was 50 μM, whilst when used together with 37.5 and 75 nM of ara-C the IC_{50} was found 38 μM and 31 μM, respectively. In the case of free Ret-OH, the IC_{50} values do not substantially change in the presence or in the absence of ara-C, being comprised in the concentration range between 35 μM and 38 μM.

When suboptimal concentrations of ara-C were used in combination with the liposomal form of ATRA and Ret-OH (Fig. 3), the IC_{50} values were again almost unchanged with respect to the corresponding VET_{100}(retinoid) without ara-C. The IC_{50} values of the VET_{100}(ATRA) plus ara-C were in fact found comprised between 0.8 μM and 1.3 μM and the VET_{100}(ATRA) alone was 0.76. In the case of VET_{100}(Ret-OH) plus ara-C, the IC_{50} were comprised between 0.4 μM and 0.6 μM (see Table III).

Taken together, the results reported in Figure 3 and Table III clearly indicate that the liposome association strongly increases the antiproliferative activity of retinoids towards both untreated K562 cells or K562 treated with suboptimal concentration of ara-C.

Differentiating Activity of Liposome-Associated Retinoids

Figures 7 and 8 show representative experiments in which the kinetics of erythroid differentiation of K562 cells was determined after different days of cell culture in the absence and in the presence of 18.25 or 37.5 nM ara-C. Accordingly with elsewhere published observations [14,19], increase of benzidine-positive cells is detectable after 3 days; differentiation was evident with 1 μM retinoids, namely ATRA and Ret-OH, plus both concentrations of ara-C, whilst low levels were obtained with retinoids alone (panels 7A and 8A). When liposomes containing 0.025 μM ATRA or Ret-OH were used, it was found that the effect of the drugs on ara-C mediated erythroid differentiation was clearly evident even at these low retinoid concentrations; high levels of differentiation were in fact reached with liposomes containing 0.025 μM retinoids plus 37.5 nM ara-C (panels 7B and 8B), while free ATRA and Ret-OH failed to induce erythroid differentiation in the presence of 37.5 nM ara-C.

The results reported in Figure 9A,C indicate indeed that when both ATRA and Ret-OH are administered as liposomal form to untreated K562 cells (filled symbols) they fail to induce increase of benzidine positive cells; on the contrary when liposomal forms of ATRA and Ret-OH are administered to K562 cells treated with suboptimal concentration of ara-C, they clearly show a synergistic activity with ara-C in inducing K562 erythroid differentiation. In these experiments, cells were treated with 37.5 nM and 75 nM ara-C in the presence of liposome-associated retinoids. The proportion of benzidine-positive cells was determined after 7 days of cell culture. It should be pointed out that also free retinoids were able to enhance the differentiating effect of suboptimal concentration of ara-C [12]; however, the liposome-associated retinoids were found active at concentration almost 10-fold lower with respect to the corresponding free drug (see a summary of the experimental data shown

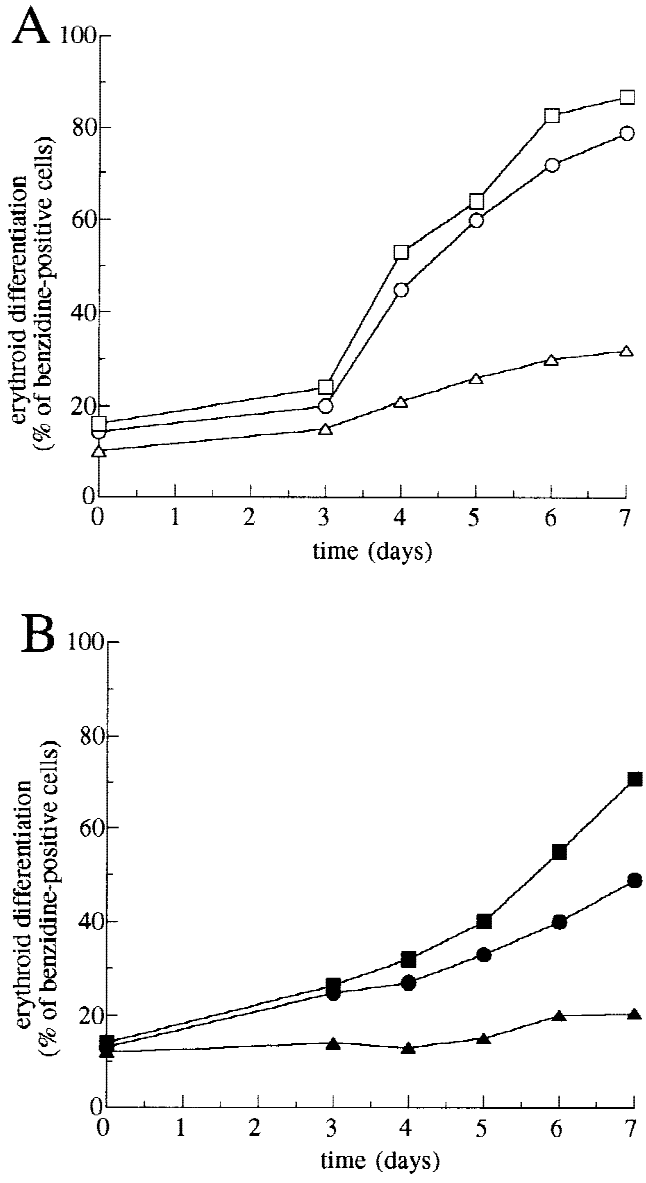

Fig. 7. Effects of ATRA and ara-C on erythroid differentiation of K562 cells. A: 1 μM free ATRA plus 18.25 nM ara-C (○); 1 μM free ATRA plus 37.5 nM ara-C (□); 1 μM free ATRA (△). B: 0.025 μM VET_{100}(ATRA) plus 18.25 nM ara-C (●); 0.025 μM VET_{100}(ATRA) plus 37.5 nM (■) ara-C; 0.025 μM VET_{100}(ATRA) (▲). The proportion of benzidine-positive (erythroid induced) K562 cells was determined after different days, as indicated.

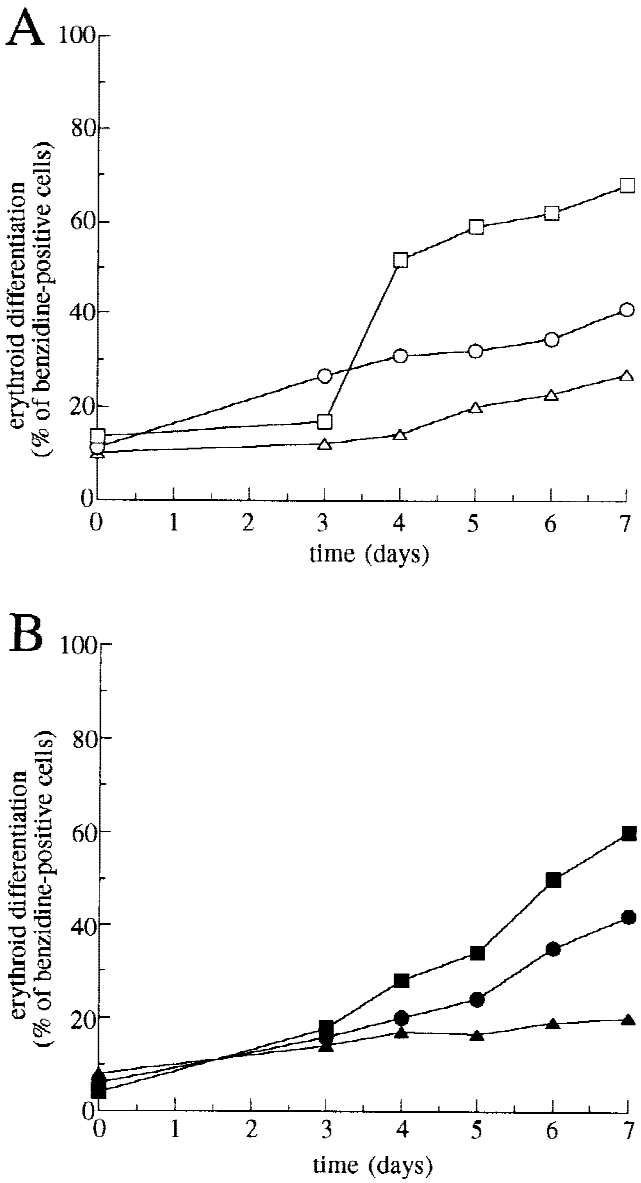

Fig. 8. Effects of Ret-OH and ara-C on erythroid differentiation of K562 cells. A: 1 μM free Ret-OH plus 18.25 nM ara-C (○); 1 μM free Ret-OH plus 37.5 nM ara-C (□); 1 μM free Ret-OH (△) B: 0.025 μM VET_{100}(Ret-OH) plus 18.25 nM ara-C (●); 0.025 μM VET_{100}(Ret-OH) plus 37.5 nM (■) ara-C; 0.025 μM VET_{100}(Ret-OH) (▲). The proportion of benzidine-positive (erythroid induced) K562 cells was determined after different days, as indicated. For comparison the proportion of benzidine-positive cells of control untreated K562 cell population is also reported (X).

in Table III). In order to exclude that the increase of benzidine-positive cell was merely due to a growth inhibition of benzidine-negative cells in Figure 9B,D we report the total number of benzidine-positive cells/ml.

Semi-solid cell cultures confirmed that erythroid-induced cells exhibit lower capability to undergo repeated cell division cycles when compared to uninduced cells; benzidine-positive K562 cell colonies were indeed reproducibly smaller in size with respect to benzidine-negative cell colonies (data not shown).

CONCLUSIONS

Differentiation therapy focuses on the development and use of specific agents designed to selectively engage

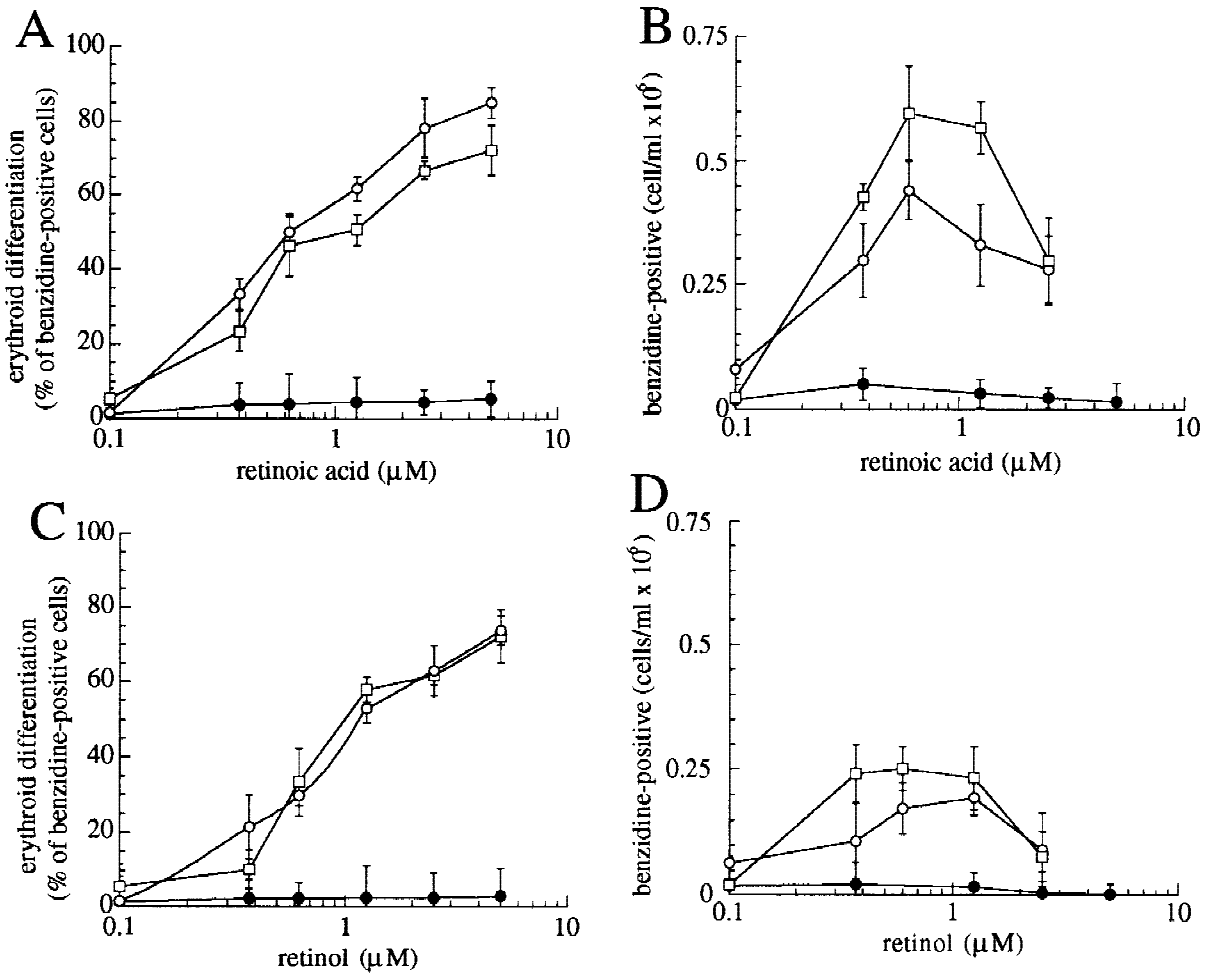

Fig. 9. Effects of liposome containing retinoids and ara-C on erythroid differentiation of K562 cells. A: Effects of VET_{100}(ATRA) on erythroid differentiation of K562 cells. K562 cells (4 × 10^4/ml) were incubated in the absence (●) or in the presence of 37.5 (□) and 75 nM (○) ara-C plus the indicated concentration of liposome-associated ATRA. Benzidine staining was performed after 7 days of treatment. B: VET_{100}(ATRA) activity on erythroid differentiation of K562 cells incubated in the absence (●) or in the presence of 37.5 (□) and 75 nM (○) ara-C. Data represent the number of benzidine-positive cells/ml after 7 days of incubation. The values reported on Y axis correspond to the activity of sole ara-C 37 and 75 nM on K562 cells. All data (A, B and C) represent the average of six independent experiments + SD. C: Effects of VET_{100}(Ret-OH) on erythroid differentiation of K562 cells. K562 cells (4 × 10^4/ml) were incubated in the absence (●) or in the presence of 37.5 (□) and 75 nM (○) ara-C plus the indicated concentration of liposome-associated Ret-OH. Benzidine staining was performed after 7 days of treatment. D: VET_{100}(Ret-OH) activity on erythroid differentiation of K562 cells incubated in the absence (●) or in the presence of 37.5 (□) and 75 nM (○) ara-C. Data represent the number of benzidine-positive cells/ml after 7 days of incubation. The values reported on Y axis correspond to the activity of sole ara-C 37 and 75 nM on K562 cells. All data (A, B and C) represent the average of six independent experiments + SD. As control, cells were also treated with the same amount of empty liposomes. This treatment did not result in any modification of cell growth or differentiation with respect to untreated cells (data not shown).

the process of terminal differentiation, leading to the eventual elimination of tumorigenic cells and rebalance of normal cellular homeostasis. Recently, a large number of studies have been focused on the encapsulation of antitumor drugs within liposomes. Liposome mediated delivery, in fact, could attenuate most of the problems associated with the systemic use of antitumor drugs such as retinoids.

The main conclusion of this paper is that the combined treatment with liposomes containing retinoids and suboptimal concentration of ara-C are effective inducers of K562 cell differentiation minimizing at the same time toxic effects. Control experiments aimed to determine possible selection of subpopulations of K562 cells suggest that the observed results are not related to toxicity and/or potential selection of induced cells. In addition,

liposomally delivered retinoids could also be proposed for differentiation therapy as effective strategy in the treatment and management of malignancy.

Furthermore, we like to mention that the identifications of new approaches to induce erythroid differentiation is crucial for experiments aimed at the development of potential therapeutic agents in hematological disorders, including β°-thalassemia [20–24]. Recently published observations demonstrate that hormones, cytotoxic agents, hemopoietic cytokines, short fatty acids are agents capable to augmenting fetal haemoglobin levels in humans [24]. For instance, hydroxyurea, erythropoietin, butyrates, and 5-azacytidine were found to induce in vitro fetal haemoglobin production when administered singularly or in combination [20–28]. With respect to this point butyric acid and 5-azacytidine have been the object of recent reports focused on in vivo treatment of β-thalassemia patients [25,26]. This is a major issue in this field, because it is well established that an increase as low as 30% of HbF production leads to a significant improvement of the clinical status. Accordingly, it is generally accepted that pharmacologic induction of expression of endogenous γ-globin genes could be considered a realistic approach to therapy of β-globin disorders [19,29]. In this respect, the demonstration that liposome delivered ATRA and Ret-OH augment the differentiation capacity of ara-C, in experiments in which these compounds are added to the cell cultures at suboptimal concentrations, is in line with a possible use of these reagents to induce expression of erythroid functions in normal human erythroid cells.

In conclusion, the results reported in the present paper are in our opinion of interest in the field of development of antitumor differentiation agents on one hand, and in the field of induction of HbF in haematological diseases on the other. Accordingly, our results should encourage preclinical studies focused on liposome-mediated delivery and tissue distribution of differentiating agent administered at very low concentrations. The fact that liposomes, ATRA, Ret-OH, and ara-C are currently used in clinical trials in a variety of human pathologies could facilitate their combined administration to patients affected by haematological diseases.

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